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## Reduced epithelial suppressor of cytokine signalling 1 in severe eosinophilic asthma

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## **TITLE PAGE**

### **Reduced epithelial suppressor of cytokine signalling 1 in severe eosinophilic asthma**

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**Take home message:**

Persistent airway eosinophilia/Th2 inflammation in severe asthma is associated with reduced epithelial SOCS1 expression.

## ABSTRACT

Severe asthma represents a major unmet clinical need. Eosinophilic inflammation persists in the airways of many patients with uncontrolled asthma, despite high dose inhaled corticosteroid therapy. Suppressors of cytokine signalling (SOCS) are a family of molecules involved in the regulation of cytokine signalling via inhibition of the JAK-STAT pathway. We examined SOCS expression in the airways of asthma patients and investigated whether this is associated with persistent eosinophilia.

Healthy controls, mild/moderate asthmatics and severe asthmatics were studied. Whole genome expression profiling, qPCR and immunohistochemical analysis were used to examine expression of SOCS1, SOCS2 and SOCS3 in bronchial biopsies. Bronchial epithelial cells were utilised to examine the role of SOCS1 in regulating IL-13 signalling *in vitro*.

SOCS1 gene expression was significantly lower in the airways of severe asthmatics compared to mild/moderate asthmatics, and was inversely associated with airway eosinophilia and other measures of Th2 inflammation. Immunohistochemistry demonstrated SOCS1 was predominantly localised to the bronchial epithelium. SOCS1 overexpression inhibited IL-13-mediated CCL26 (eotaxin-3) mRNA expression in bronchial epithelial cells.

Severe asthma patients with persistent airway eosinophilia and Th2 inflammation have reduced airway epithelial SOCS1 expression. SOCS1 inhibits epithelial IL-13 signalling, supporting its key role in regulating Th2 driven eosinophilia in severe asthma.



## INTRODUCTION

Asthma is one of the most common chronic conditions affecting approximately 300 million people worldwide.[1] Most patients with asthma achieve adequate disease control with bronchodilator and inhaled corticosteroid therapy.[2] However, approximately 10% of patients suffer from severe uncontrolled asthma despite the use of high dose inhaled corticosteroids, and in many cases systemic corticosteroids. This cohort of asthma patients represent a significant unmet clinical need.[3]

Asthma is a heterogeneous disorder thus current research is endeavouring to identify and characterise distinct phenotypes, particularly in severe disease.[4, 5] Both clinical and molecular phenotyping have been utilised to describe populations of asthma patients in terms of inflammatory biology.[6-8]

A key feature in many patients with severe asthma is persistent eosinophilic airway inflammation despite high dose corticosteroid treatment.[9, 10] The major group of cytokines implicated in driving this inflammation are the Th2-associated cytokines, namely IL-4, IL-5 and IL-13. IL-13 is a key cytokine contributing to the pathophysiological features of asthma including airway hyper-responsiveness, mucus hypersecretion and airway remodelling and IL-13 overexpression persists in a subgroup of patients with severe asthma despite high dose steroid treatment.[10, 11]

Th2 cytokines mediate their actions via the JAK-STAT pathway and cytokine signalling is tightly regulated by several molecules including the suppressors of cytokine signalling (SOCS). SOCS are a family of 8 proteins, comprising SOCS1-7 and CIS, which inhibit cytokine signalling through inhibition of the JAK-STAT pathway in a negative feedback manner.[12] SOCS1 was the first of the SOCS family to be discovered.[13] *In vitro* cell cultures and *in vivo* animal models have demonstrated that SOCS1 is a negative regulator of

Th2-dependent pathways, achieved by inhibition of the phosphorylation of STAT6.[14, 15] SOCS2 inhibits growth hormone and insulin-like growth factor [16] and has been shown to regulate the expression of other SOCS molecules.[17] Recently, we have shown that SOCS2 knockout mice have an increased susceptibility to Th2 mediated airway inflammation compared to wild type mice, due to elevated SOCS3 levels.[18] SOCS3 mRNA expression is reported to be increased in the peripheral T cells of asthma patients [19, 20] and constitutive SOCS3 expression in the T cells of mice resulted in exaggerated lung inflammation in a mouse model of asthma.[20]

The principal aim of this study was to examine SOCS1, SOCS2 and SOCS3 expression in the airways of healthy controls, mild/moderate asthmatics and severe asthmatics and to explore the relationship between SOCS expression and persistent eosinophilic inflammation within the airway.

## METHODS

### Patient cohort

Data from this well characterised cohort of subjects has been published previously.[10, 21, 22] Healthy controls (n=17), mild/moderate asthmatics (n=29) and severe asthmatics (n=18) were recruited from two UK specialist asthma centres, Belfast City Hospital and Glenfield Hospital, Leicester (demographic characteristics shown in Table 1). Clinical assessment protocols ensured patients had severe asthma as defined by current ERS/ATS guidelines.[23] Further details of asthma patients in the study, including asthma treatment, can be found in the online supplementary file (Table E1). This study was approved by Research Ethics Committees of both institutions and written informed consent obtained from all subjects (see online supplementary file).

**Table 1.** Demographic characteristics of study participants.

	Healthy Controls (n=17)	Mild-Moderate Asthmatics (n=29)	Severe Asthmatics (n=18)	P value
Age (years)	31.82 ± 11.74	31.86 ± 9.93	46.94 ± 10.70	<0.0001
Sex, male/female (n)	7 / 10	17 / 12	12 / 6	ns
Duration of asthma (years)	N/A	14.59 ± 13.76	19.39 ± 15.01	ns
Asthma onset (age)	N/A	17.28 ± 13.29	27.56 ± 18.99	0.03
FEV1 (L/min)	3.69 ± 0.62	3.48 ± 0.73	2.45 ± 0.60	<0.0001
FEV1 (% predicted)	102 ± 12.01	92.44 ± 16.78	75.17 ± 16.79	<0.0001
FEV/FVC (%)	82.11 ± 11.57	73.43 ± 11.01	61.3 ± 13.48	0.0017
Mean asthma exacerbations/year	N/A	2.2 ± 0.83	5.26 ± 4.14	<0.0001
Regular inhaled steroid n (%)	N/A	20 (69)	18 (100)	ns
BDP equivalent (µg)	N/A	400 (0 - 800)	1600 (800 - 1700)	<0.0001
Systemic steroids n (%)	N/A	0 (0)	12 (67)	<0.0001
Prior smoking n (%)	1 (6)	6 (21)	7 (39)	ns

Beclomethasone Dipropionate (BDP) equivalent is expressed as median  $\pm$  IQR. Age, duration of asthma, asthma onset, FEV1 (L/min), FEV1 (% predicted), FEV1/FVC (%), mean asthma exacerbations/year are expressed as mean  $\pm$  standard deviation. Between group comparisons were calculated using ANOVA with Bonferroni's multiple comparisons test or Kruskal Wallis with Dunn's multiple comparison test where appropriate, t-test when only asthma groups compared or Chi-squared for categorical variables; ns = not significant.

### **Bronchoscopy**

Each participant underwent bronchoscopy and biopsy specimens were taken from the right middle lobe and lower lobe carinae. Biopsies were immediately placed in RNA preservative (Ambion, USA) or fixed overnight before embedding in glycol methacrylate (GMA) as described previously.[24]

### **Gene expression analyses**

Bronchial biopsies were homogenised, RNA extracted and amplified for Agilent two colour Whole Human Genome 4x44k gene expression microarray analysis as previously described.[25] Th2 score was calculated as described previously and details can be found in the online supplementary file.[8, 10, 25] TaqMan® Gene Expression Assays (Applied Biosystems, USA) were used as per manufacturer's instructions. GAPDH was utilised as the house keeping gene. qPCR values were calculated using the  $\Delta\Delta C_t$  method with relative gene expression calculated as  $-\Delta\Delta C_t$ . [26] Further details are available in the online supplementary file.

### **Immunohistochemistry (IHC)**

Sections of bronchial biopsies were cut at 2µm, immunostained as described previously [24] and counterstained with Mayer's haematoxylin. Negative controls, using an isotype and/or omitting primary antibody, were employed. The lamina propria was measured in biopsy sections using a computer analysis system (LAS V3.7 software, Leica, Germany). For enumerating cellular inflammatory infiltrate, individual positively stained nucleated cells were counted and expressed per mm<sup>2</sup> of tissue. Figure E1 in the online supplementary file shows exemplar micrographs of inflammatory cell staining. IHC was additionally employed to determine localisation of SOCS proteins in patient biopsies. Full details of antibodies are available in the online supplementary file.

### **Bronchial epithelial cell cultures**

The BEAS-2B cell line (ATCC, USA) was cultured in Dulbecco's Modified Eagle Medium supplemented with 5% foetal calf serum and 1% Penicillin/Streptomycin. Primary bronchial epithelial cells (PBECs) were obtained from healthy volunteers. This work was approved by Research Ethics Committee as detailed in the online supplementary file and written informed consent was gained from all subjects.[22] PBECs were cultured in airway epithelial cell basal medium containing a supplement pack (Promocell, Germany). Human recombinant IL-13 (R&D Systems, USA) was used at 50 ng/ml. *In vitro* experiments were carried out a minimum of 3 times.

### **Transfections**

Myc-tagged full-length SOCS1 in a pcDNA3 plasmid, along with the empty vector, were a generous gift from Prof. A. Yoshimura (Keio University School of Medicine, Japan). BEAS-2B cells were plated in antibiotic-free media and transfected using Lipofectamine 2000

(Invitrogen, UK). PBECs were transfected by electroporation using the NEON transfection system (Life Technologies, UK). Media was changed 6 hours post-transfection and cell stimulations were carried out 24 hours post-transfection.

### **Western blotting**

Cells were lysed and 30 µg of protein was immunoblotted with antibodies against SOCS1, SOCS2, SOCS3, c-Myc and  $\gamma$ -tubulin as described in the online supplementary file.

### **Statistical analyses**

GraphPad, Prism 5 software was used to analyse data. Data is presented as median (IQR) where appropriate. Kruskal-Wallis test, Mann–Whitney U test, Student *t*-test and Spearman's Rank correlations were used for statistical tests unless otherwise stated. PBEC data was transformed (log10) prior to statistical analysis.

## **RESULTS**

### **Increased Th2 inflammatory measures in study participants with asthma.**

Peripheral blood eosinophil numbers and blood IgE levels were significantly elevated in mild/moderate asthmatics and severe asthmatics as compared to healthy controls (Figure 1A and 1B respectively). Quantification of eosinophils using IHC showed a trend of increasing numbers of eosinophils in the lamina propria of bronchial biopsies with increasing asthma severity (healthy control, mild/moderate asthma and severe asthma group median values were 4.59, 9.70 and 12.93 respectively,  $p=0.07$ ) (Figure 1C). There were no significant differences noted in the number of neutrophils or mast cells between the study groups (Table E2 in the online supplementary file). The previously generated gene expression microarray Th2

signature from asthma patients is a composite measure of IL-13 induced epithelial genes.[8, 10, 25] There was also a trend for increasing Th2 biopsy signature values with increasing asthma severity ( $p=0.057$ ). There was a significant positive correlation between the intensity of the Th2 biopsy signature and the number of eosinophils/mm<sup>2</sup> of lamina propria of the asthma patients (Figure 1E). No correlation was observed between neutrophils or mast cells and the Th2 signature (Figure E2 in the online supplementary file).

### **Decreased SOCS1 mRNA expression in bronchial biopsies from severe asthmatics compared to mild/moderate asthmatics.**

Gene expression of SOCS1, SOCS2 and SOCS3 mRNA in bronchial biopsies of healthy controls, mild/moderate asthmatics and severe asthmatics was assessed using qPCR. SOCS1 expression was significantly decreased in the bronchial biopsies of severe asthmatics compared to mild/moderate asthmatics (Figure 2A). However, there were no differences observed in SOCS2 and SOCS3 gene expression levels between groups (Figure 2B and 2C respectively). Of note SOCS1 mRNA was decreased in the airways of asthma patients receiving high dose inhaled corticosteroids compared those receiving low dose inhaled corticosteroids but there was no difference in SOCS2 or SOCS3 expression (Figure E3).

### **SOCS1 gene expression is decreased in patients with persistent Th2 inflammation and eosinophilia in the airways.**

Significant negative correlations were observed between SOCS1 gene expression and both the Th2 biopsy signature and eosinophil numbers in the lamina propria of bronchial tissue in asthma patients (Figure 3A and 3B respectively). There were no significant correlations identified between SOCS2 or SOCS3 and Th2 biopsy signature or eosinophil numbers in bronchial biopsies (Figure E4 in the online supplementary file). To further examine the

relationship between SOCS1 expression and tissue eosinophilia, subjects were dichotomised by the number of eosinophils in the lamina propria of their bronchial biopsies (eosinophil counts were dichotomised around the median value for eosinophils/mm<sup>2</sup> within the lamina propria). Participants with high eosinophils ( $\geq 9.2$  eosinophils/mm<sup>2</sup> within the lamina propria) were found to have significantly decreased levels of SOCS1 when compared to those presenting with low eosinophils ( $< 9.2$  eosinophils/mm<sup>2</sup> within the lamina propria) (Figure 3C). Subjects dichotomised into eosinophil high and eosinophil low subsets again showed no difference in SOCS2 or SOCS3 expression between the two groups (Figure E5 in the online supplementary file). As CCL26 is highly induced in response to type 2 inflammation the relationship between this chemokine and SOCS1 was explored. As anticipated a negative correlation was observed indicating patients with low SOCS1 expression fail to inhibit the Th2 induced CCL26 (Figure 3D).

### **Localisation of SOCS proteins in bronchial biopsies.**

To examine the cellular localisation of SOCS protein expression, IHC was employed to stain for SOCS1, SOCS2 and SOCS3 in the bronchial biopsies. SOCS1 was localised predominantly to the bronchial epithelium and observed in the differentiated pseudo-columnar cells rather than the basal cells (Figure 4 shows representative images of A, negative control staining, and SOCS1 staining of B, healthy controls, C, mild/moderate asthmatics and D, severe asthmatics). There was minimal SOCS2 staining evident throughout the bronchial biopsies with only a small number of infiltrating inflammatory cells in the lamina propria staining positive for SOCS2 (Figure E6 in the online supplementary file). SOCS3 was found to be present both in the epithelium and in large, mononuclear/macrophage like cells in the lamina propria (Figure E6 in the online supplementary file).



### **Inhibition of IL-13 induced CCL26 mRNA in bronchial epithelial cell SOCS1 overexpression models.**

Due to the localisation of SOCS1 to the bronchial epithelium and its potential role in inhibiting Th2 inflammation within the severe asthmatic airway, the BEAS-2B cell line was initially used to further characterise the role of SOCS1 in IL-13 signalling in the bronchial epithelium. We examined induction of SOCS1 mRNA in BEAS-2B cells after IL-13 (50 ng/ml) exposure and found it was rapidly up-regulated within 2 hours of stimulation and remained elevated over the 24 hour time course. SOCS2 and SOCS3 mRNA were not induced in response to IL-13 treatment (Figure 5A). We then investigated the expression of SOCS1, SOCS2 and SOCS3 protein in response to IL-13 stimulation observing SOCS1 but not SOCS2 or SOCS3 induction (Figure 5B). CCL26 (eotaxin-3) mRNA is upregulated *in vitro* by IL-13 stimulation and in bronchial biopsies of asthma patients with high levels of Th2 inflammation in their airways.[25] CCL26 mRNA upregulation was confirmed in BEAS2-B cultures stimulated with IL-13 (Figure 5C). To investigate the role of SOCS1 in the regulation of IL-13 induced CCL26, SOCS1 was overexpressed in BEAS-2B cells using a Myc-tagged SOCS1 plasmid. Transfection with the SOCS1 plasmid lead to overexpression of SOCS1 in the cells compared to the empty vector control (Figure 5D). Next, cells were transfected with either the empty vector or the Myc-tagged SOCS1 plasmid for 24 hours, then stimulated with IL-13 for a further 24 hours. A significant decrease in CCL26 mRNA was observed in the SOCS1 overexpressing cells (Figure 5D).

We then wished to confirm these observations in primary bronchial epithelial cells (PBECs). PBECs were stimulated with IL-13 (50 ng/ml) over a 24 hour time course. SOCS1 mRNA was again rapidly up-regulated 2 hours after stimulation and remained elevated over the 24 hour time course and SOCS2 and SOCS3 mRNA were not induced (Figure 6A). As anticipated, CCL26 mRNA was also induced in IL-13-stimulated cells (Figure 6B). PBECs

were transfected using electroporation (transfection efficiencies were routinely greater than 55% as shown in Figure E7) and again, overexpression of SOCS1 reduced IL-13 induction of CCL26 mRNA compared to empty vector control (Figure 6C).

## DISCUSSION

Severe asthma affects up to 10% of all asthma patients, but the underlying mechanisms causing persistent eosinophilic inflammation, despite high dose inhaled corticosteroid treatment, are yet to be fully elucidated.[10, 27] SOCS molecules have been shown to regulate Th2 cytokine signalling both *in vitro* and in *in vivo* mouse models of allergic airway inflammation, however, it is unclear if similar regulatory mechanisms exist in human asthma.

In this study we investigated, for the first time, the expression of SOCS1, SOCS2 and SOCS3 in the airways of healthy controls, mild/moderate asthmatics and severe asthmatics. We show that patients with severe asthma and persistent eosinophilic airway inflammation have decreased epithelial SOCS1 expression compared to patients with milder more stable asthma. This reduced SOCS1 was inversely associated with an IL-13-dependent Th2 gene signature and tissue eosinophilia, which appears paradoxical, as one would anticipate IL-13 signalling to be associated with increased SOCS1 expression in an attempt to ‘switch off’ IL-13 driven responses. It is worth commenting that baseline SOCS1 expression in healthy subjects is low (and not different from severe asthmatics), presumably reflecting the absence of pro-inflammatory cytokine stimuli and thus no requirement for the negative feedback regulator. The key difference is that in the severe asthma population with persistent Th2 signalling and eosinophilia, the failure of SOCS1 up-regulation is inappropriate.

This effect appears specific for SOCS1, as we identified no differences in SOCS2 or SOCS3 expression in relation to disease severity, Th2 signature, or eosinophilic inflammatory infiltrate. SOCS2 and SOCS3 have been implicated in animal models of asthma and SOCS molecules can interact with each other. For example, SOCS2 has been shown to regulate the expression of SOCS1 and SOCS3 via direct interactions [17] and SOCS2 knockout mice were shown to have elevated levels of SOCS1 and SOCS3 in T cells.[18] However, our data

examining the expression of SOCS molecules in human asthma suggests a specific dysregulation of SOCS1 in severe asthma with persistent Th2 inflammation despite high dose inhaled corticosteroids. One limiting factor of our study is the cross sectional design, therefore we cannot comment on the dynamic changes in SOCS expression. However, the pattern of specific SOCS1 down-regulation was consistent across severe asthma patients with persistent eosinophilia thus suggesting that it is unlikely to be a transient feature.

We utilised immunohistochemistry to examine localisation of SOCS proteins and better inform our subsequent *in vitro* mechanistic analysis. SOCS1 was found to be localised predominantly to the bronchial epithelium of the airway tissue. This is consistent with previous data, whereby mice challenged intra-tracheally with IL-13 displayed SOCS1 induction localised to the airway epithelium.[15]

*In vitro* studies and *in vivo* animal models have shown SOCS1 attenuates Th2 inflammation. Local airway induction of SOCS1 in response to IL-13 administration in an *in vivo* mouse model was associated with suppression of eotaxin levels.[15] In another study, an OVA-induced airway inflammation model was utilised and SOCS1<sup>-/-</sup> IFN- $\gamma$ <sup>-/-</sup> mice had increased eosinophilic infiltration in the lungs and elevated Th2 cytokines compared to IFN- $\gamma$ <sup>-/-</sup> control mice.[14] These animal studies support our data in human asthma whereby reduced SOCS1 expression in severe asthma is also associated with airway eosinophilia and an IL-13-dependent Th2 gene signature, suggesting epithelial SOCS1 has a central role in regulating IL-13 signalling in the airway and airway eosinophilia. Indeed high SOCS1 expression in airway eosinophils has been reported to inhibit their response to the IL-5 family cytokines.[28] Therefore SOCS1 deficiency in eosinophils in the airway of severe asthma patients may cause increased sensitivity to IL-5 cytokine signaling leading to enhanced eosinophil survival and pathologies in the airway.

IL-13 is a Th2 cytokine that has been implicated in human asthma and is elevated in severe asthma subjects with persistent eosinophilia.[10, 29] IL-13 signals via the STAT6 pathway and the absolute requirement of SOCS1 in attenuating Th2 inflammation via inhibition of STAT6 phosphorylation has been observed in SOCS1 deficient mouse embryonic fibroblasts.[15, 30] As SOCS1 was localised to the bronchial epithelium of bronchial biopsies, we wished to examine the role of SOCS1 in regulating IL-13 epithelial signalling *in vitro* using the bronchial epithelial BEAS-2B cell line and primary bronchial epithelial cells (PBECs). CCL26 (eotaxin-3) expression was examined as a downstream readout of IL-13 signalling as previous studies have found it to be one of the most highly upregulated genes in the airways of asthma patients with Th2 inflammation, it is IL-13 dependent and a potent eosinophil chemoattractant.[31, 25, 32] Cells stimulated with IL-13 showed SOCS1 mRNA was rapidly induced but this was not the case for SOCS2 or SOCS3. This selective induction of SOCS1 further supports its role in inhibiting IL-13 driven Th2 inflammation and is consistent with our patient data showing no relationship between SOCS2 or SOCS3 expression and the IL-13 dependent gene signature or airway eosinophilia. As anticipated, and consistent with other systems, CCL26 mRNA was induced in BEAS-2B cells and PBECs in response to IL-13. Consistent with role of SOCS1 regulating IL-13 signalling, when SOCS1 was over-expressed in BEAS-2B and PBEC cultures, IL-13 induction of CCL26 was inhibited. We have previously reported in this same cohort of severe asthmatics, that patients with an elevated IL-13 dependent-Th2 gene signature, have increased numbers of IL-13<sup>+</sup> cells infiltrating the epithelium.[10] Taken together, these patients with severe asthma, despite being on high dose inhaled corticosteroid treatment, have persistent airway eosinophilia, increased intra-epithelial IL-13<sup>+</sup> cells, persistent IL-13 signalling in the airway (Th2 gene signature) and decreased SOCS1 expression. This suggests a breakdown in regulation of IL-13 by SOCS1 in these patients and it will be of interest to assess whether SOCS1 expression

is modified upon IL-13 inhibition, for example, after treatment with IL-13 antagonists such as those currently in phase 2/3 clinical trials.[28, 33, 34]

A study assessing functional variants of SOCS1 within a population of adult Japanese asthma patients found a significant association between the SOCS1 promoter polymorphism, -1478CA< del, and adult asthma. It is suggested this promoter polymorphism leads to increased SOCS1 and inhibition of interferons (IFNs) leading to higher susceptibility to virus-induced asthma exacerbations.[35] Another study has recently shown that increased nuclear SOCS1 in asthmatic bronchial epithelium suppresses rhinovirus induction of innate IFNs.[36] However, our data in severe asthmatics would suggest that reduced SOCS1 in the epithelium is associated with persistent eosinophilia, which is a major risk factor for exacerbations in this population.[37]

In summary, we have investigated the expression of SOCS1, SOCS2 and SOCS3 within the airways of healthy controls, mild/moderate asthmatics, and severe asthmatics. We found that SOCS1 was significantly decreased in the airways of severe asthma patients when compared to mild/moderate asthma patients. Subjects with persistent eosinophilia and increased Th2 inflammation within the airway have decreased epithelial SOCS1 expression. *In vitro* investigation of BEAS-2B and primary bronchial epithelial cell cultures showed that SOCS1 was induced in response to IL-13 stimulation and SOCS1 overexpression led to reduced CCL26 mRNA induction in response to IL-13 stimulation. Further work will focus on why there is an inappropriate failure to upregulate SOCS1 in the epithelium of patients with severe asthma despite persistent Th2 signalling in the airway.

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## Figure legends

**Figure 1. Increased Th2 inflammatory measures in study participants with asthma.** **A**, Peripheral blood eosinophils and **B**, blood IgE levels increased with increasing asthma severity. **C**, and **D**, showed a trend of increasing eosinophil numbers in the lamina propria and increasing Th2 signature values with increasing asthma severity. **E**, Eosinophil numbers in the lamina propria positively correlated with Th2 biopsy signature (n=40). Black circles represent mild/moderate asthma patients and white circles represent severe asthma patients. Kruskal-Wallis and Spearman's Rank tests were employed for statistical analysis. Graphs show median and IQR. \*\* P<0.01, \*\*\* P<0.001.

**Figure 2. Decreased SOCS1 mRNA expression in bronchial biopsies from severe asthmatics compared to mild/moderate asthmatics.** **A**, SOCS1, **B**, SOCS2 and **C**, SOCS3 mRNA expression was analysed in biopsy homogenates from healthy controls (n=17), mild/moderate asthmatics (n=29) and severe asthmatics (n=18) using qPCR. Values represent  $-\Delta\Delta Ct$ . Kruskal-Wallis test was employed for statistical analysis. Graphs show median and IQR. \* P<0.05.

**Figure 3. SOCS1 gene expression is decreased in patients with persistent Th2 inflammation and eosinophilia in the airways.** **A**, SOCS1 and the Th2 biopsy signature (n=47) and **B**, SOCS1 and eosinophil numbers in the lamina propria (n=40) showed a significant negative correlation. **C**, SOCS1 expression was decreased in subjects with high airway eosinophilia ( $\geq 9.2$  eosinophils/mm<sup>2</sup> lamina propria, n=26) compared to those with low airway eosinophilia ( $< 9.2$  eosinophils/mm<sup>2</sup> lamina propria, n=26). **D**, SOCS1 and CCL26 expression in the airway showed a significant negative correlation (n=44). Black circles represent mild/moderate asthma patients and white circles represent severe asthma patients.

Mann–Whitney U test and Spearman's Rank tests were employed for statistical analysis. Graphs show median and IQR. \* $P < 0.05$ , \*\*  $P < 0.01$ , \*\*\*  $P < 0.001$ .

**Figure 4. Localisation of SOCS1 protein predominantly to the bronchial epithelium.**

**A**, shows negative control for SOCS1 staining. GMA embedded bronchial biopsy sections from **B**, healthy controls, **C**, mild/moderate asthmatics and **D**, severe asthmatics were stained for SOCS1 using immunohistochemistry. Staining showed localisation of SOCS1 to the bronchial epithelium as depicted by the brown staining. Representative images show negative control staining and epithelial SOCS1 biopsy staining (x200 and x400 respectively).

**Figure 5. SOCS1 overexpression in BEAS-2B cultures inhibited IL-13 induced CCL26 mRNA.**

**A**, Cells stimulated with IL-13 (50 ng/ml) over 24 hours showed upregulation of SOCS1 mRNA but not SOCS2 or SOCS3 mRNA (n=3). **B**, Cells stimulated with IL-13 (50 ng/ml) over 24 hours showed upregulation of SOCS1 protein but not SOCS2 or SOCS3 protein. **C**, Cells stimulated with IL-13 (50 ng/ml) over 24 hours showed upregulation of CCL26 mRNA. **D**, Cells were transfected with an empty vector or SOCS1 overexpression plasmid then probed for SOCS1 and the plasmid Myc-tag to confirm SOCS1 overexpression. Cells were then treated with an empty vector or SOCS1 overexpression plasmid for 24 hours, stimulated with IL-13 for a further 24 hours then CCL26 mRNA was measured using qPCR. Graphs show median and IQR. Mann–Whitney U test employed for statistical analysis (n=4). \* $P < 0.05$ .

**Figure 6. SOCS1 overexpression in PBEC cultures inhibited IL-13 induced CCL26 mRNA.**

**A**, Cells stimulated with IL-13 (50 ng/ml) over 24 hours showed upregulation of SOCS1 mRNA but not SOCS2 or SOCS3 mRNA (n=5). **B**, Cells stimulated with IL-13 (50

ng/ml) over 24 hours showed upregulation of CCL26 mRNA. **C**, Cells were transfected with an empty vector or SOCS1 overexpression plasmid for 24 hours and stimulated with IL-13 for a further 24 hours then CCL26 mRNA was measured using qPCR. Graphs show median and IQR. Kruskal-Wallis and Students *t*-test were employed for statistical analysis (n=3). \*P<0.05, \*\* P<0.01.



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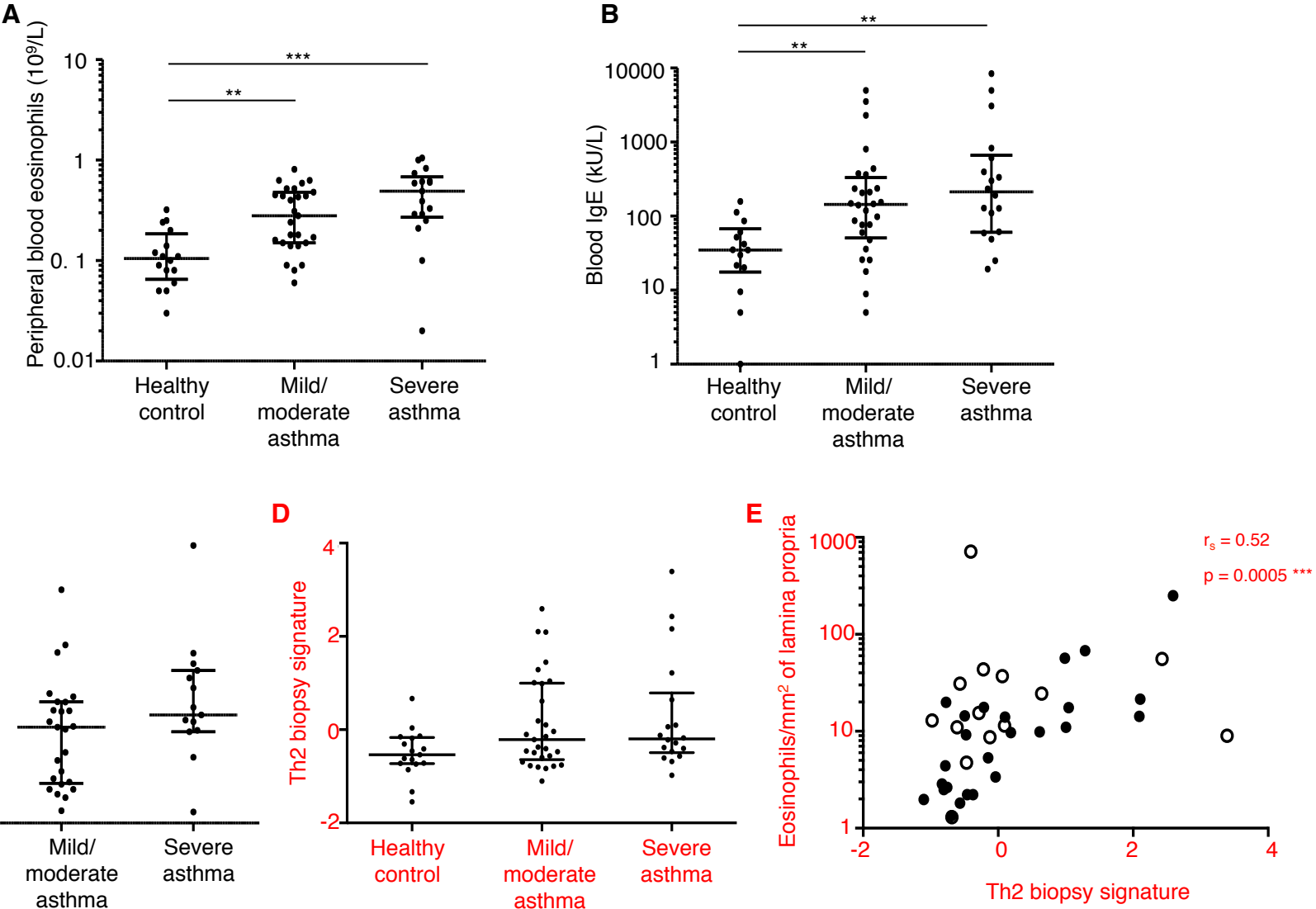


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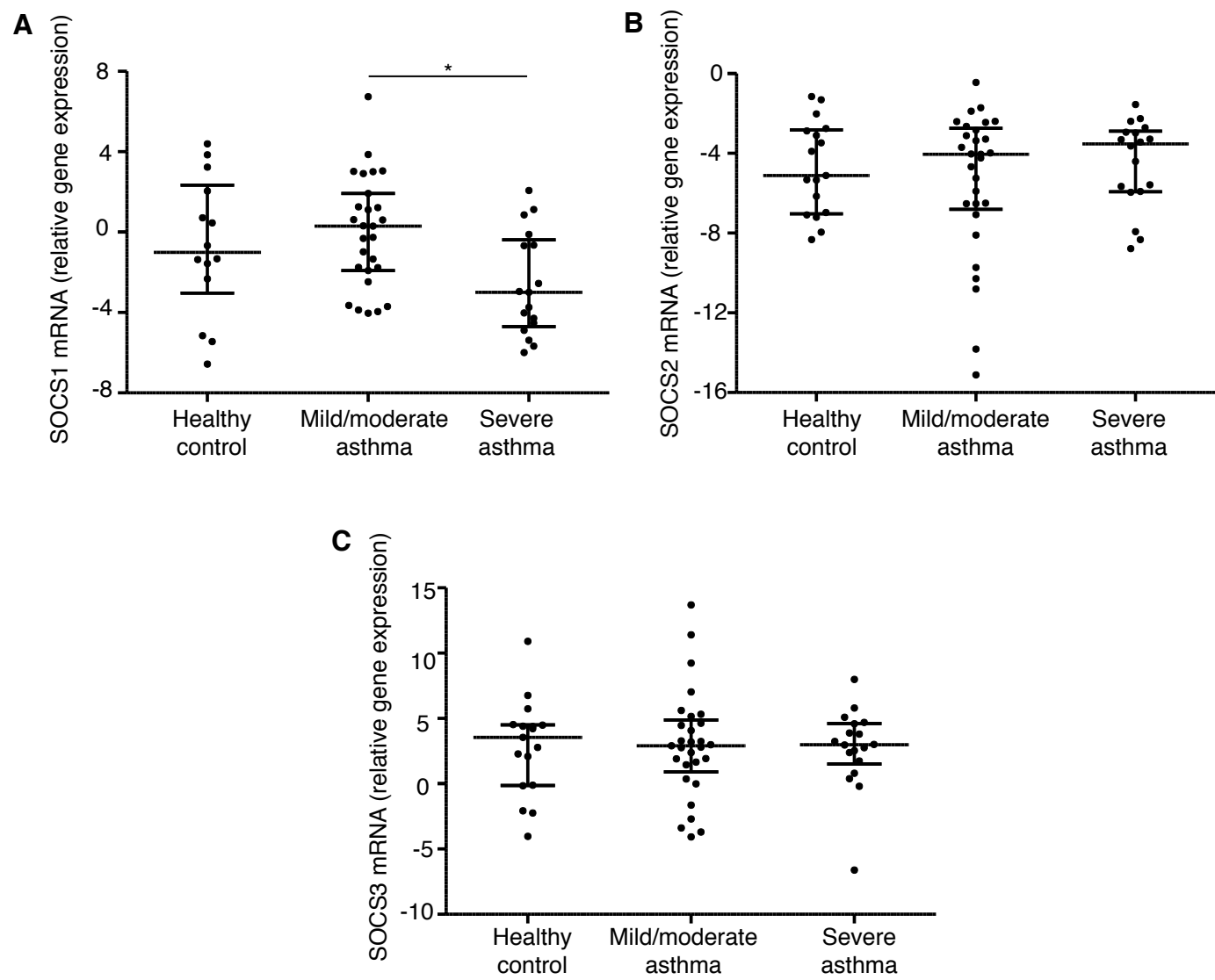
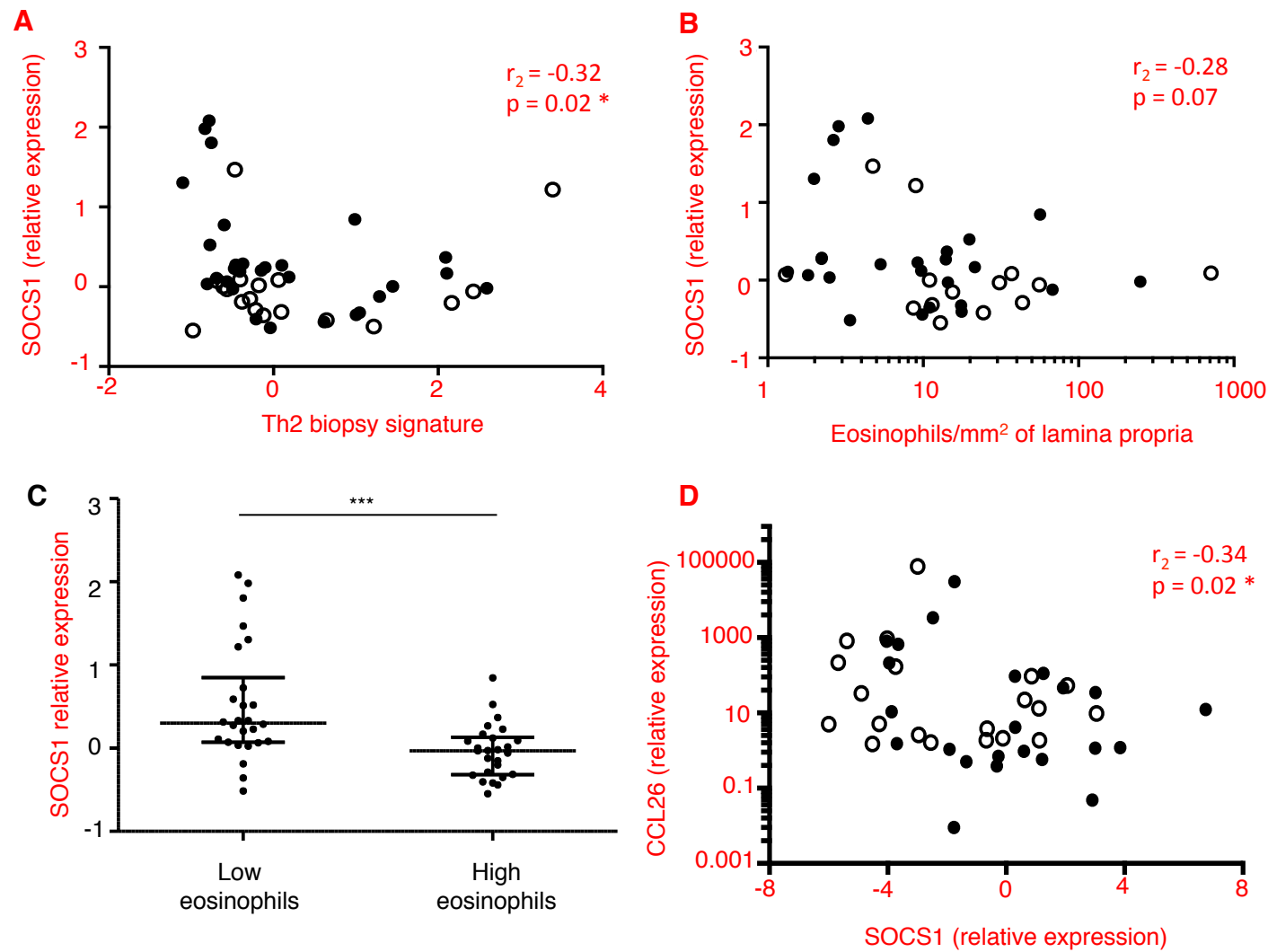
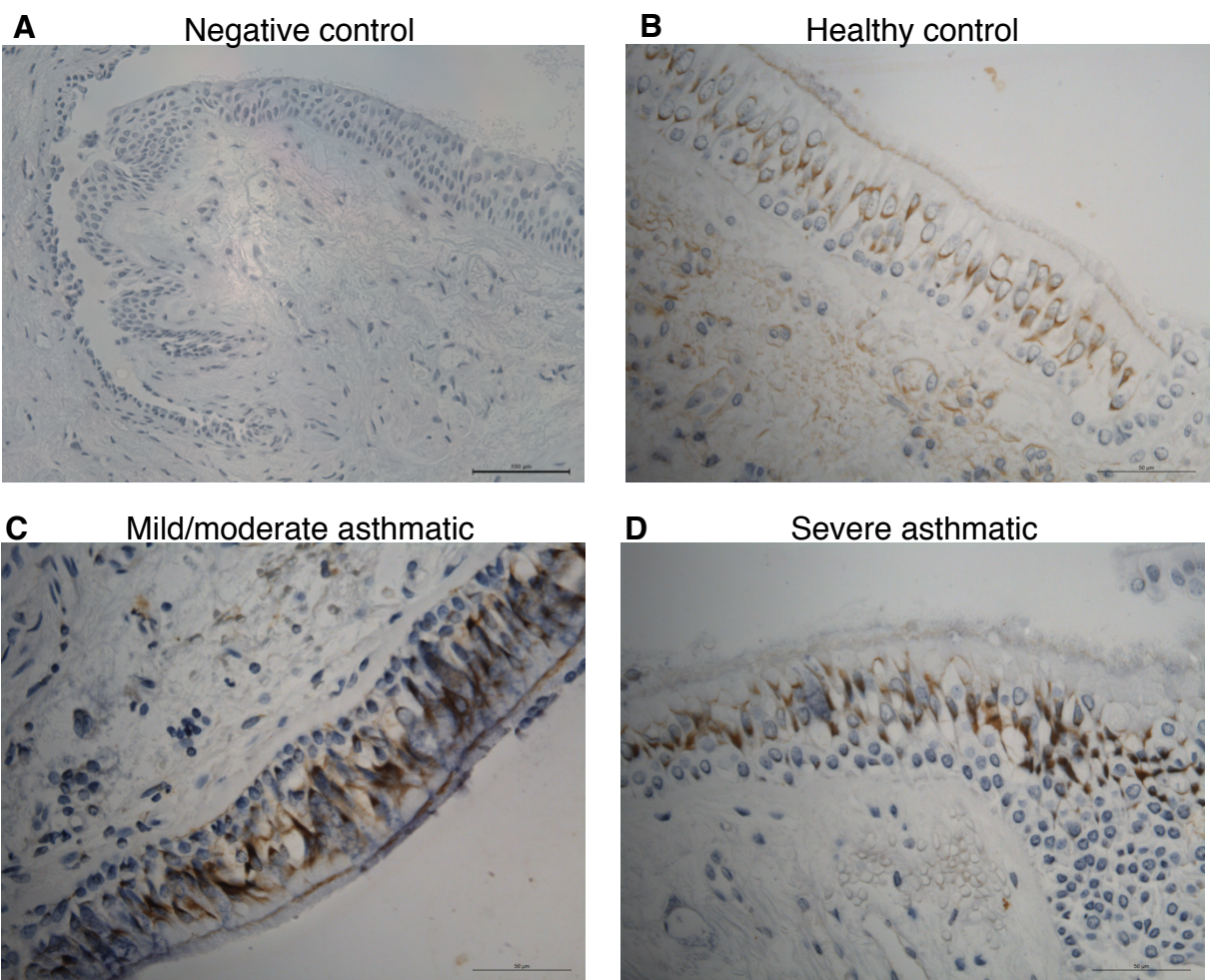


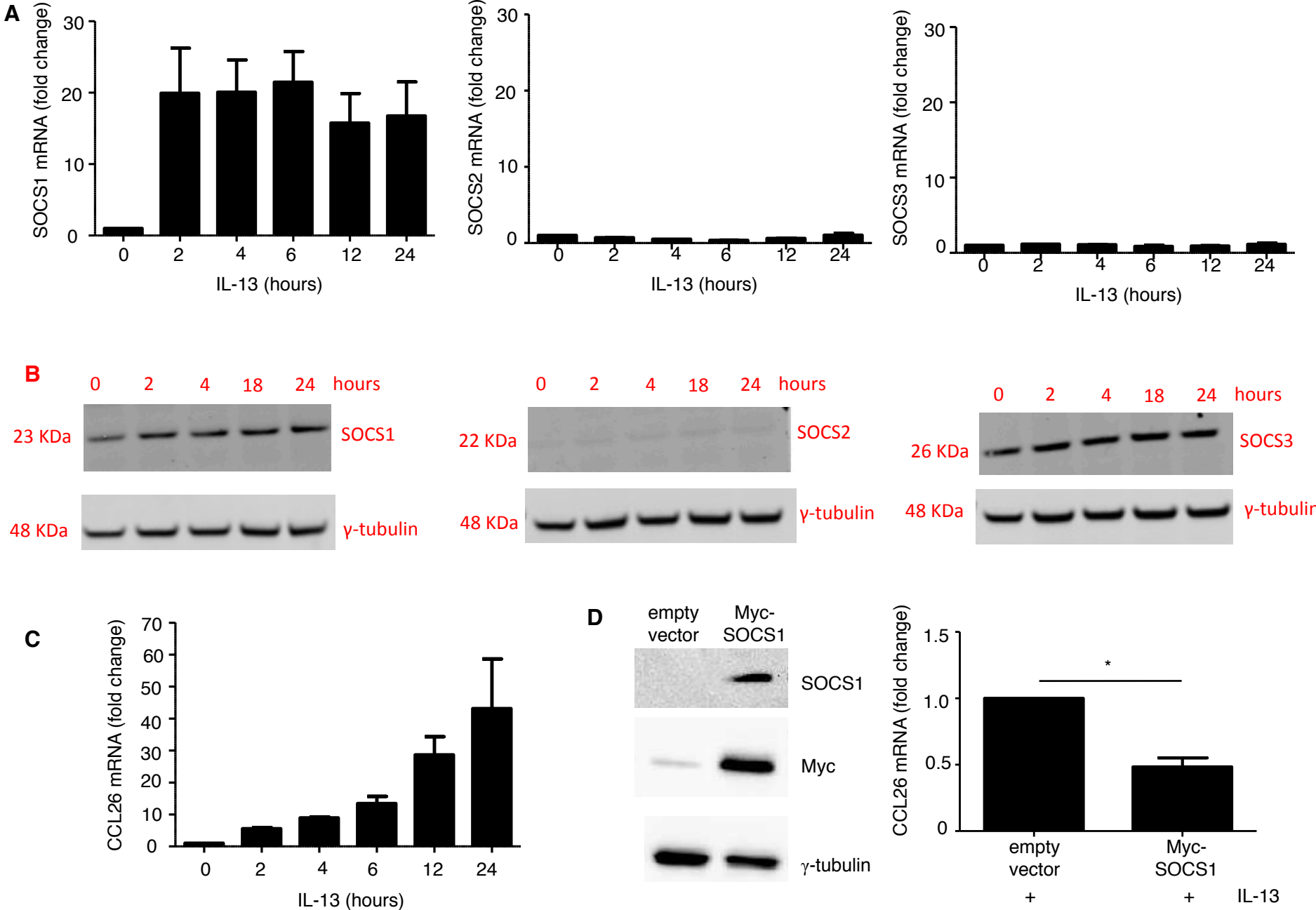
Figure 3.



**Figure 4.**



**Figure 5.**



**Figure 6.**

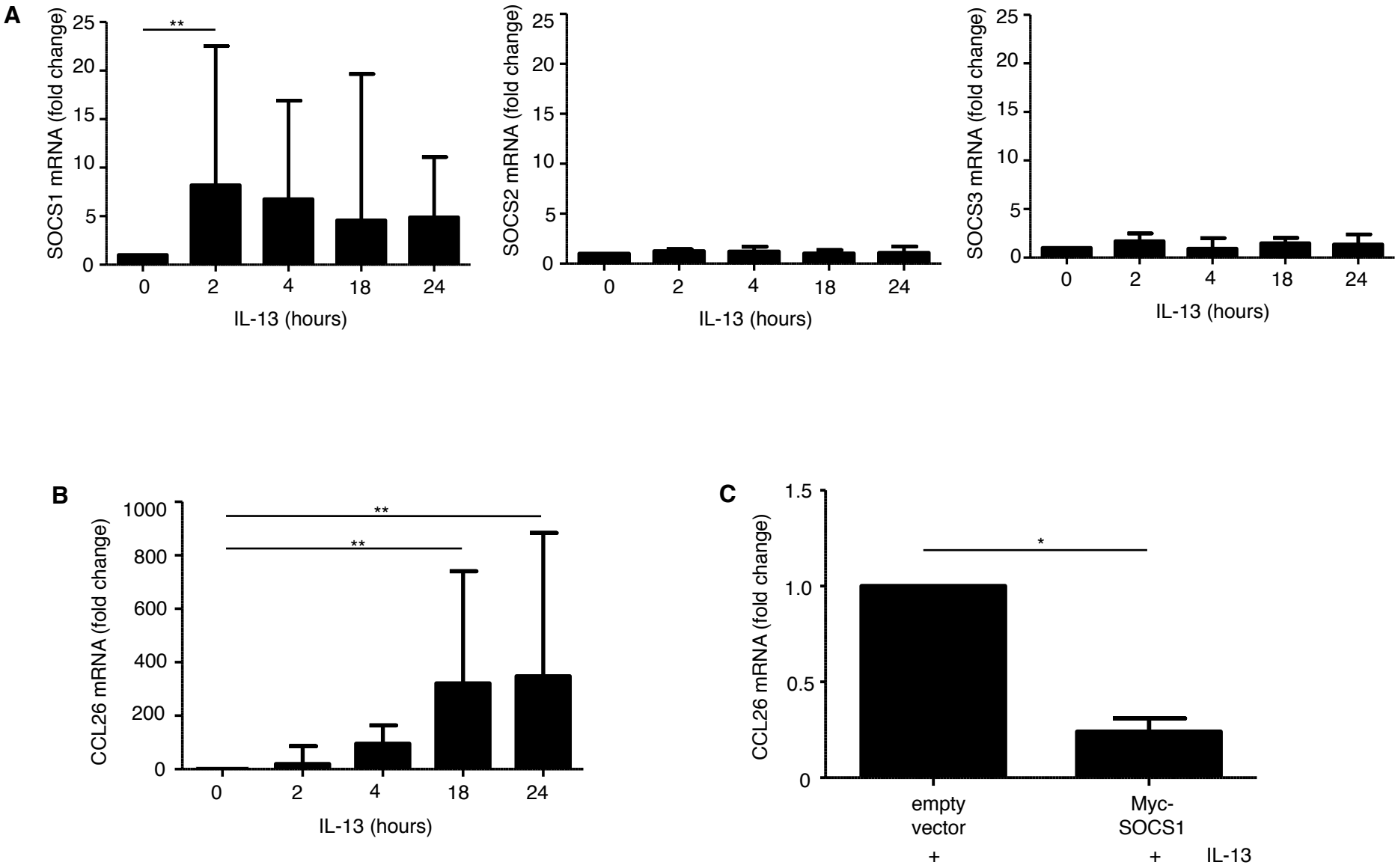
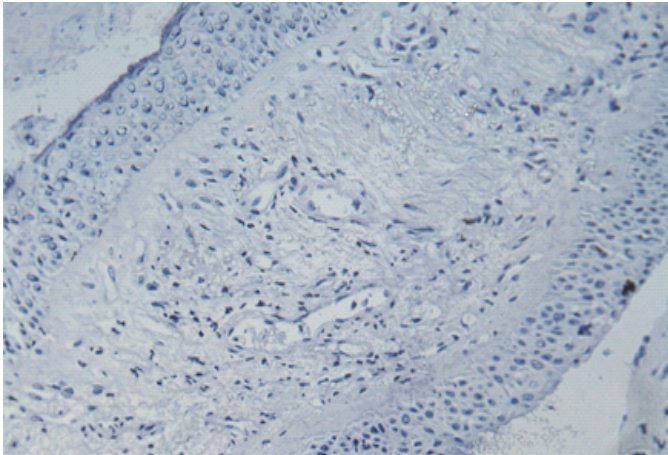


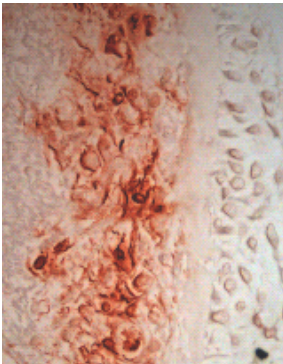
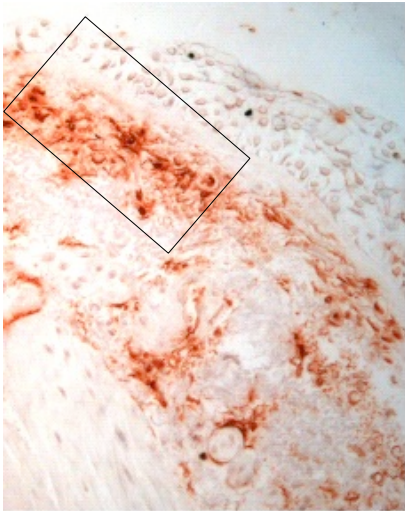


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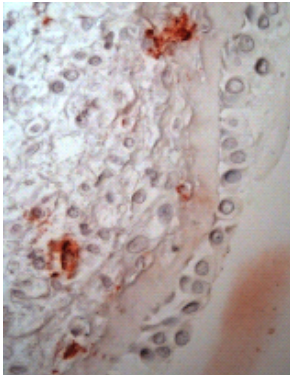
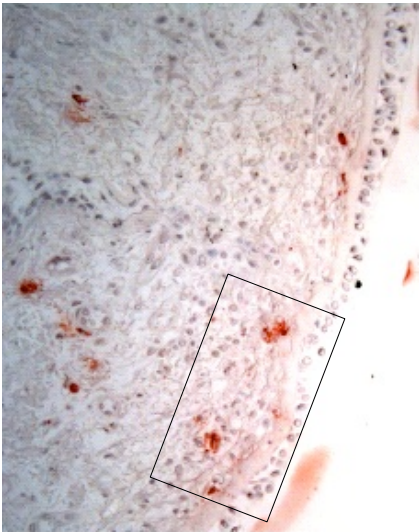
A



B



C



D

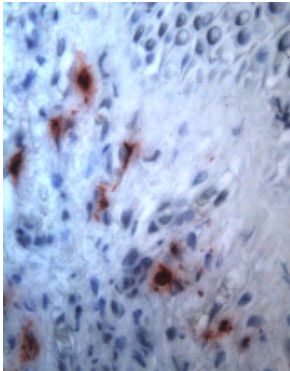
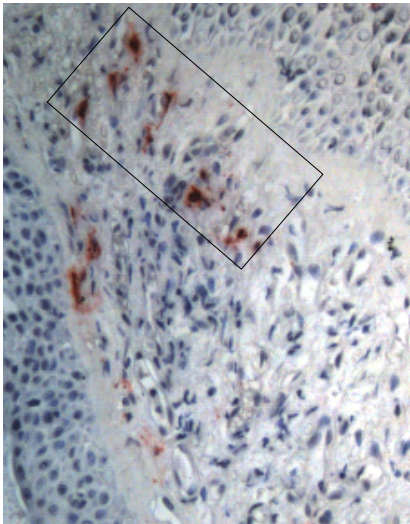


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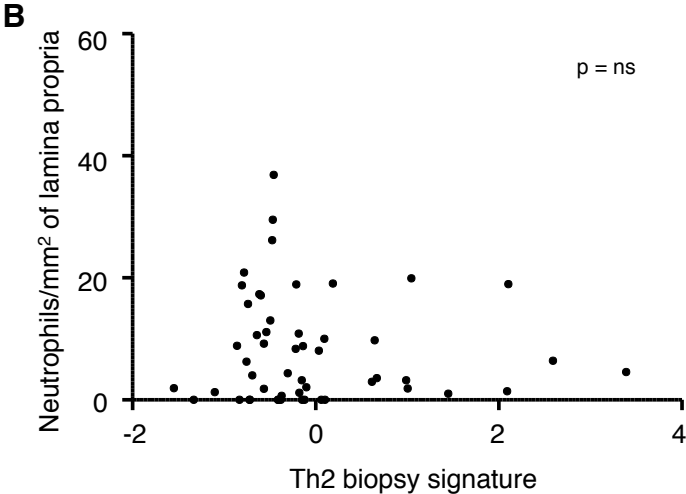
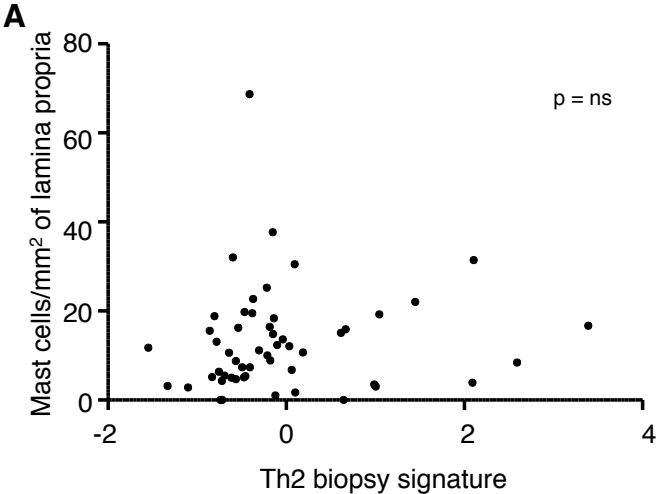




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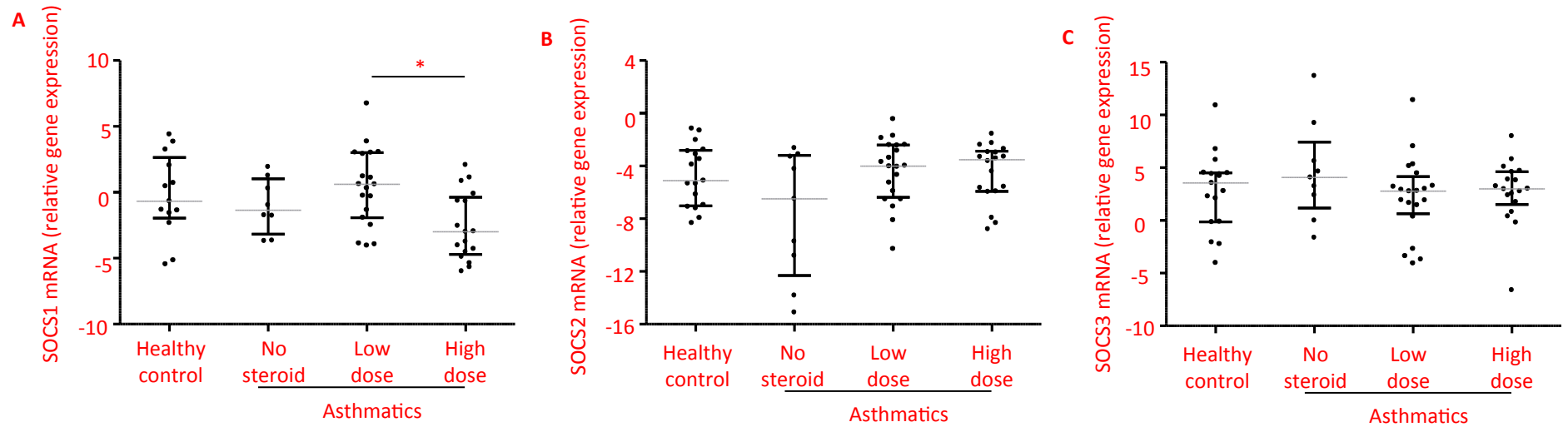


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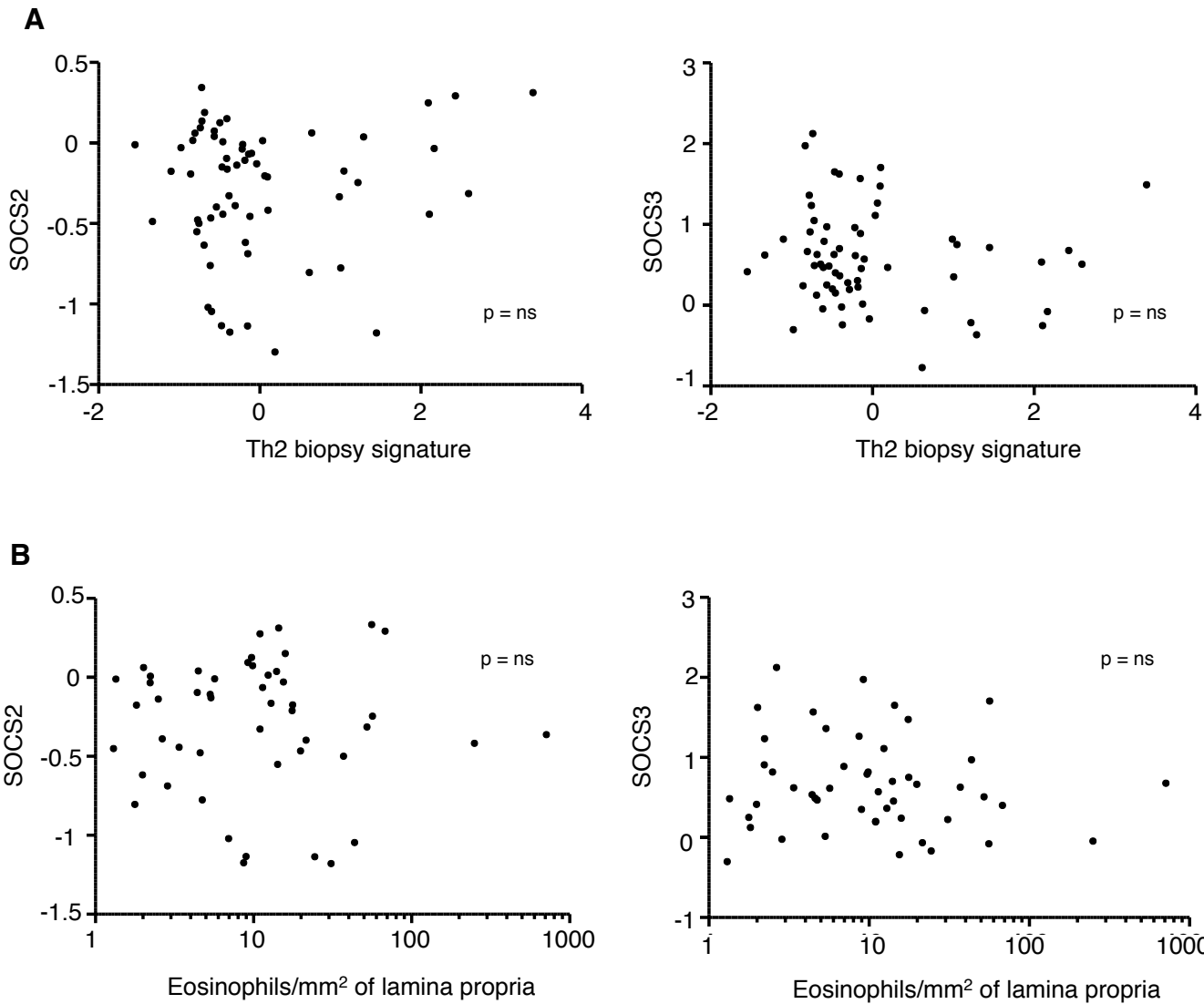


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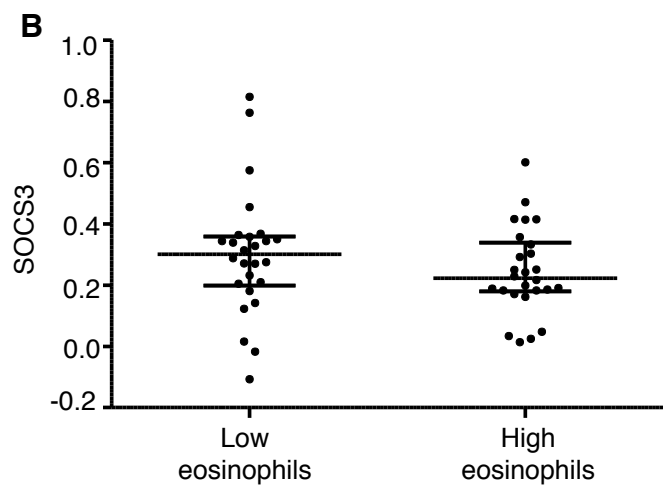
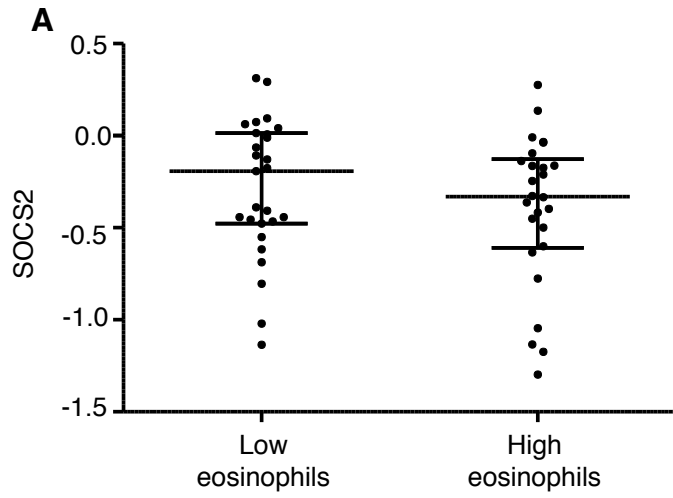


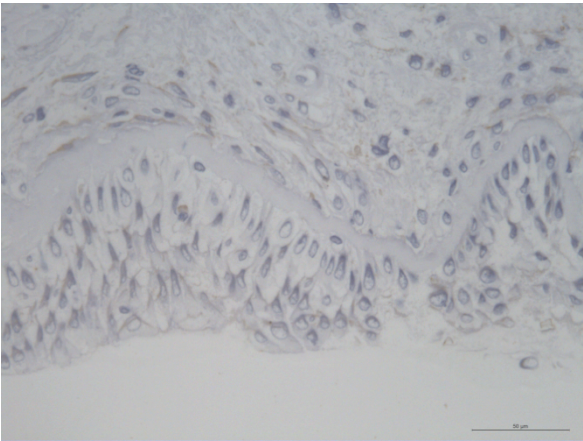
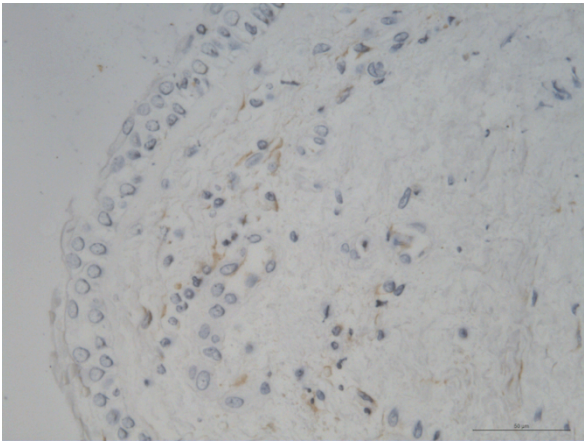
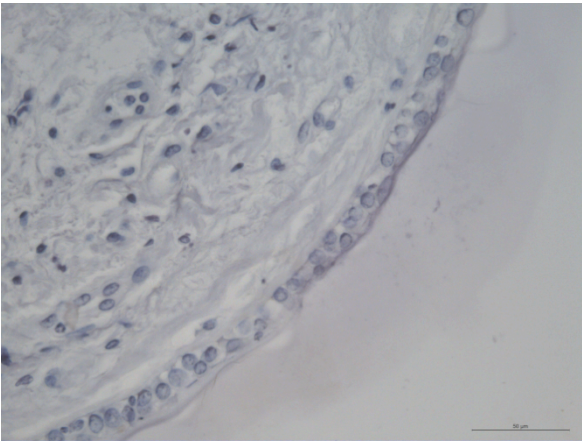
Figure E6.

Healthy control

Mild/moderate asthmatic

Severe asthmatic

SOCs2



SOCs3

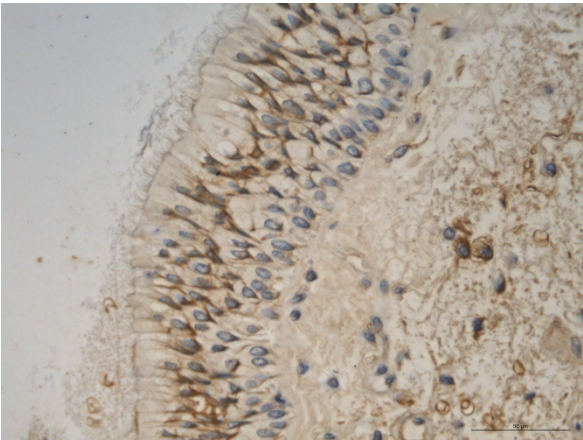
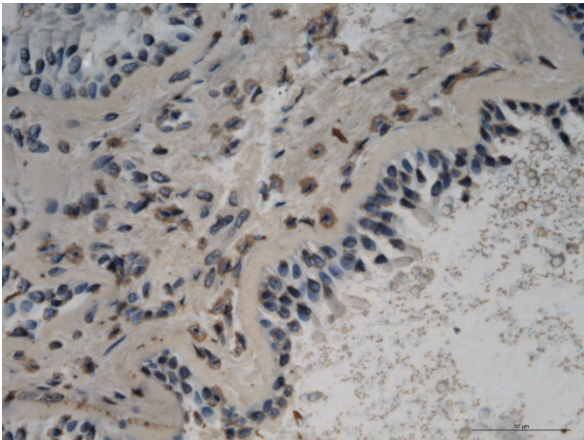
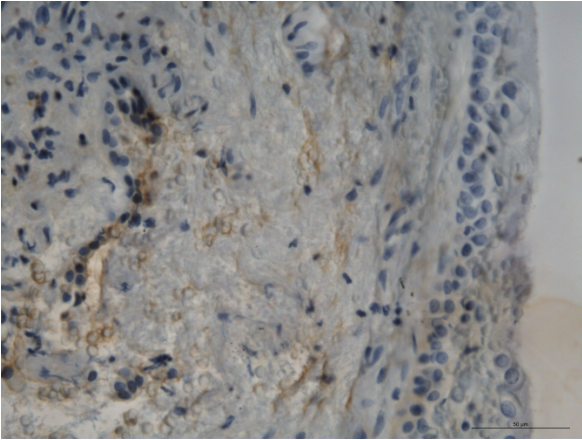
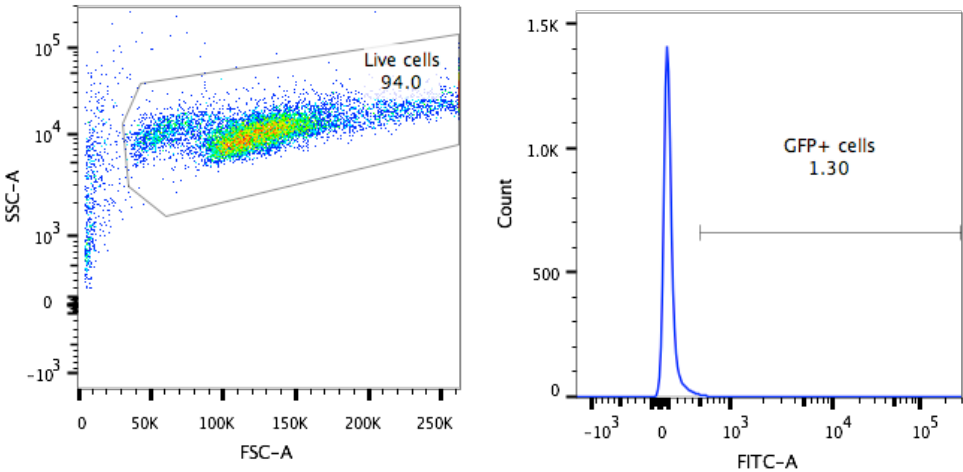


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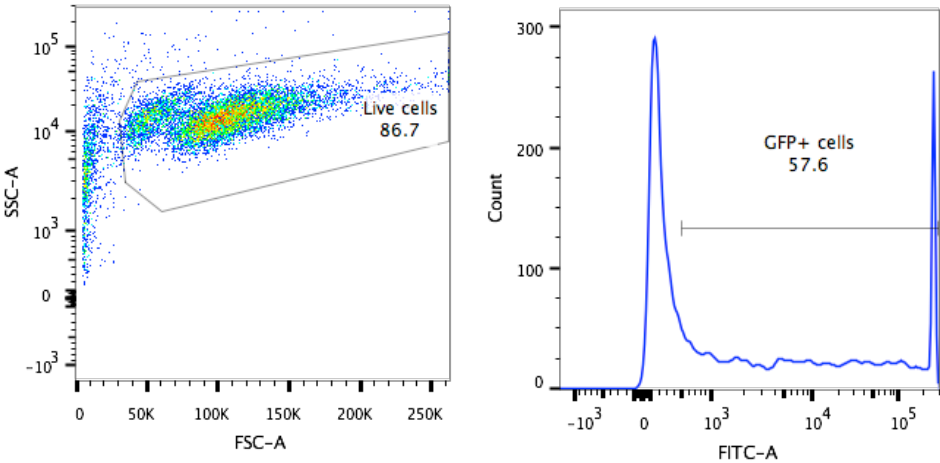
A

Mock transfection



B

GFP tagged plasmid



## **Online supplementary files**

### **Reduced epithelial suppressor of cytokine signaling 1 in severe eosinophilic asthma**

Emma Doran, PhD<sup>a</sup>, David F Choy, BSc<sup>b</sup>, Aarti Shikotra, PhD<sup>c</sup>, Claire A Butler, PhD<sup>a</sup>, Declan M O'Rourke, MD<sup>d</sup>, James A Johnston, PhD<sup>c</sup>, Adrien Kissenpfennig, PhD<sup>a</sup>, Peter Bradding, DM<sup>c</sup>, Joseph R Arron, MD, PhD<sup>b</sup>, and Liam G Heaney, MD<sup>a</sup>.

## **Online methods**

### **Patient cohort**

This study was approved by the Research Ethics Committee of both institutions (Belfast - Office of Research and Ethics Committee of Northern Ireland reference 06/NIR02/114 and 09/NIR02/51 and Leicester - Leicestershire, Northamptonshire, & Rutland Research Ethics Committee reference 04/Q2502/74). Mild to moderate asthma patients and healthy volunteers were recruited from hospital clinic or by advertisement.

### **Bronchoscopy**

All participants were current non-smokers with no upper or lower respiratory tract infection in the 6 weeks prior to bronchoscopy and were clinically stable and receiving their usual medication at the time of bronchoscopy.

### **Th2 Biopsy Signature**

The Th2 score was calculated by using a generalized procedure. [25] Thirty-eight signature genes were selected based on array features corresponding to Th2 signature genes with Entrez gene annotation that were previously observed to be upregulated in Th2-high subjects. If individual Entrez genes mapped to multiple probes, the probe with the greatest interquartile range was retained, and any others were removed. Gene expression values were mean centered. Missing Th2 signature gene set values (5/2622 [0.19%]) were substituted by using mean replacement. Principal component analysis was conducted on these expression values, and PC1 (which retained 39.5% of the variance of the signature gene set) was used as the Th2 score.

### **Gene expression analyses**

TaqMan® Gene Expression Assays (Applied Biosystems, USA) were purchased and used as per manufacturer's instructions for SOCS1 (Hs00864158\_g1), SOCS2 (Hs00919620\_m1), SOCS3 (Hs02330328\_s1), CCL26 (Hs00171146\_m1). GAPDH (4333764F) was utilized as a housekeeping gene. Universal Human Reference cDNA (#639654, Clontech, CA) was utilized as a calibrator sample for bronchial biopsy analysis and untreated cells were used as the calibrator for *in vitro* studies.

### **Immunohistochemistry (IHC)**

The following primary antibodies were used: mouse monoclonal anti-mast cell tryptase clone AA1 (1:1000 dilution; Dako, UK), mouse monoclonal anti-eosinophil major basic protein clone BMK-13 (0.4 µg/mL; Monosan, The Netherlands), mouse monoclonal anti-neutrophil elastase clone NP57 (1:1000 dilution; Dako, UK), rabbit polyclonal anti-SOCS1 (1 µg/mL; Zymed Laboratories, Invitrogen, UK), rabbit polyclonal anti-SOCS2 (1:100 dilution; Cell Signaling Technologies, USA), mouse monoclonal anti-SOCS3 clone 1B2 (40 µg/mL; Millipore, USA) and appropriate isotype controls (Dako, UK). The following secondary antibodies were used: polyclonal rabbit anti-mouse (1:300 dilution) and polyclonal swine anti-rabbit (1:300 dilution), both biotinylated antibodies were purchased from Dako, Denmark.

### **Western blotting**

The following primary antibodies were used: rabbit polyclonal anti-SOCS1 (1:1000 dilution, Cell Signalling Technology, USA), rabbit polyclonal anti-SOCS2 (1:1000 dilution, Cell Signalling Technology, USA), rabbit polyclonal anti-SOCS3 (1:1000 dilution, Cell Signalling Technology, USA), anti-c-Myc (9E10) antibody (1:1000 dilution, Santa Cruz



Biotechnology, USA) and anti- $\gamma$ -tubulin (1:5000 dilution, Sigma-Aldrich, UK) according to manufacturer's instruction.

The following secondary antibodies were used: Goat anti-rabbit HRP and goat anti-mouse HRP utilised at a 1:10,000 dilution (Bio-Rad, UK).

**Table E1.** Treatment and exacerbation records of asthma patients.

Disease	ICS.dose. BDP.equiv.	OCS.mg. prednisone.d.equiv.	Severe exacerbations requiring rescue steroids in past year	Additional controller treatment
MMA	0	0	0	
MMA	0	0	0	
MMA	0	0	0	
MMA	0	0	0	
MMA	0	0	0	
MMA	0	0	0	
MMA	0	0	0	
MMA	1000	0	1	
MMA	0	0	0	
MMA	0	0	0	
MMA	400	0	0	
MMA	500	0	0	Salmeterol
MMA	800	0	2	Eformoterol
MMA	500	0	0	
MMA	100	0	0	Eformoterol
MMA	800	0	0	Eformoterol bd
MMA	400	0	0	
MMA	1000	0	0	Salmeterol
MMA	400	0	0	
MMA	800	0	2	Eformoterol, Theophylline
MMA	400	0	0	Eformoterol
MMA	500	0	0	
MMA	200	0	0	Salmeterol
MMA	400	0	0	Salmeterol
MMA	400	0	3	Salmeterol
MMA	400	0	0	Salmeterol
MMA	800	0	0	Eformoterol, Monteleukast Theophylline
MMA	800	0	0	Eformoterol, Monteleukast
MMA	800	0	3	Eformoterol
SA	1600	0	1	Eformoterol
SA	1600	10	10	Eformoterol, Monteleukast Theophylline
SA	800	Kenalog 80 mg monthly	12	Eformoterol, Monteleukast Theophylline
SA	2000	0	3	Salmeterol
SA	2000	0	0	salmeterol
SA	1600	7.5	1	Eformoterol, Monteleukast Ipratropium
SA	2400	0	4	Eformoterol
SA	1600	20	12	Eformoterol, Theophylline
SA	2400	4	12	Eformoterol,
SA	800	5	0	Eformoterol, Monteleukast Theophylline, Ipratropium
SA	1200	5	0	Eformoterol, Theophylline
SA	400	5	2	Eformoterol, Theophylline
SA	800	10	2	Eformoterol
SA	2000	10	4	Salmeterol, Monteleukast Theophylline
SA	2000	20	6	Salmeterol, Theophylline
SA	1600	0	3	Eformoterol, Theophylline
SA	1600	10	2	Eformoterol
SA	1600	0	5	Eformoterol

Mild moderate asthma patient (MMA), severe asthma patient (SA), Beclomethasone Dipropionate (BDP).

**Table E2.** Quantification of mast cells and neutrophils in the lamina propria of bronchial biopsy tissue.

Cell type	Healthy controls	Mild/Moderate asthmatics	Severe asthmatics	P value
Mast cell	13.13 (6.97 – 18.78)	10.71 (5.10 – 19.63)	7.35 (4.27 – 15.57)	ns
Neutrophil	8.43 (1.58 – 19.68)	3.24 (1.08 – 12.06)	6.33 (0.16 – 14.50)	ns

IHC was carried out to identify mast cell and neutrophil numbers in bronchial biopsies obtained from healthy controls, mild/moderate and severe asthmatics. The area of lamina propria was measured in each biopsy, positively stained cells were counted and expressed as the number of positive cells/mm<sup>2</sup> of tissue. Kruskal-Wallis tests were employed for statistical analysis. Table shows median and IQR. ns = not significant.

### **Online figure legends**

#### **Figure E1. Identification of eosinophils, mast cells and neutrophils in bronchial biopsies.**

Bronchial biopsies were embedded in glycol methacrylate (GMA), sectioned and stained using IHC. **A**, Representative mouse IgG1 isotype control staining. Antibodies targeting **B**, eosinophilic major basic protein, **C**, mast cell tryptase and **D**, neutrophil elastase were utilised for the identification of each cell type. Representative images show biopsy staining captured at x200 and inserts show biopsy image captured at x400.

#### **Figure E2. Mast cells and neutrophils in the lamina propria do not correlate with biopsy**

**Th2 signature.** **A**, The number of mast cells in the lamina propria and **B**, the number of neutrophils in the lamina propria of bronchial biopsies do not correlate with the biopsy Th2 signature. Spearman's Rank test was employed for statistical analysis. ns = not significant.

#### **Figure E3. Significantly reduced SOCS1 mRNA expression observed in bronchial biopsies from severe asthmatics receiving high dose steroid treatment.**

**A**, SOCS1, **B**, SOCS2 and **C**, SOCS3 mRNA levels in bronchial biopsies were measured in healthy controls (n=17), asthmatics receiving no steroid (n=9), asthmatics receiving low dose steroid treatment of <1000 µg/day of BDP equivalent (n=19) and asthmatics receiving high dose steroid treatment of >1000 µg/day of BDP equivalent (n=18). Data is expressed as median and inter-quartile range and a Kruskal-Wallis test with Dunn's multiple comparison test was employed for statistical analysis. \* P<0.05.

#### **Figure E4. SOCS2 and SOCS3 bronchial biopsy expression do not correlate with biopsy**

**Th2 signature or eosinophil numbers.** **A**, SOCS2 and SOCS3 biopsy expression do not correlate with Th2 biopsy signature and **B**, SOCS2 and SOCS3 biopsy expression do not correlate with eosinophil numbers in the lamina propria. Spearman's Rank test was employed for statistical analysis. ns = not significant.

**Figure E5. SOCS2 and SOCS3 bronchial biopsy expression are not different between patients with high and low biopsy eosinophil numbers.** There was no difference observed in **A**, SOCS2 and **B**, SOCS3 expression in subjects with high airway eosinophilia ( $\geq 9.2$  eosinophils/mm<sup>2</sup> lamina propria, n=26) compared to those with low airway eosinophilia ( $< 9.2$  eosinophils/mm<sup>2</sup> lamina propria, n=26). Mann–Whitney U test was employed for statistical analysis. Graphs show median and IQR.

**Figure E6. Localisation of SOCS2 and SOCS3 protein in bronchial biopsies.** Sections were cut from GMA embedded bronchial biopsies of healthy controls, mild/moderate asthmatics and severe asthmatics. Sections were stained for SOCS2 and SOCS3 using immunohistochemistry. Representative images shown (x400).

**Figure E7. Measuring transfection efficiency in PBEC cultures.** a) PBECs were transfected by electroporation (2 pulses, 1400 V, 20 ms) for mock transfection or with GFP tagged plasmid to measure transfection efficiency. Cells were harvested for FACS analysis 18 hours post transfection. Mock transfected cells were utilized as the experimental control.